CHEMISTRY OF AFTER-RIPENING, GERMINATION, AND SEEDLING DEVELOPMENT OF JUNIPER SEEDS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 284

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Introduction

In a previous paper the author (17) reported the microchemical and physical changes accompanying the after-ripening, germination, and seedling development of juniper seeds. This work was undertaken with the idea of studying the physiological and chemical changes occurring in the fats during the after-ripening and the seedling development of seeds of *Juniperus virginiana*.

Historical

As early as 1842 DE SAUSSURE (3), while studying the germination of hemp, madia, and rape seeds, discovered two important results that accompany the germination of oily seeds. He concluded that oily seeds during germination absorb a larger volume of oxygen than the volume of carbon dioxide given off, and that the percentage of reserve oil decreases and the percentage of sugar increases during germination. Part of DE SAUSSURE'S work was later confirmed by the investigations of HELLRIEGEL (9) and others.

SACHS (20) in 1859 studied the transformation of oil in many seeds, and concluded that starch was directly derived from oils. PETERS (19) held this same view, but FLEURY (4) denied the constant appearance of starch, and stated that sugar appeared first. The latter investigator was the first to note the probable appearance of organic acids during germination.

MÜNTZ (16) was the first to discover the presence of free fatty acids in germinating oily seeds. While working on rape, poppy, and radish seeds he found that the oil gave rise to free fatty acids. He also noted that this free fatty acid increased several fold during germination. Although the presence of glycerine was not

demonstrated, he concluded that the oil was split up into free fatty acid and glycerine.

GREEN (7), while investigating the reserve products of *Ricinus communis* seeds during germination, discovered the enzyme lipase. He proved that this enzyme was capable of splitting the glycerides of this seed into glycerine and fatty acid. This investigator concluded that glycerine gave rise to sugar, while the fatty acids gave rise to vegetable acids. He also demonstrated the presence of a trypsin-like enzyme, which digested proteids. In a later paper (8) he continued the same investigation and worked especially on the lecithin and sugar content. The lecithin was thought of as being derived from the oils, phosphatic globoids, and proteins. In this paper he discussed the improbability of sugar being formed from glycerine.

In 1895 LECLERC DU SABLON (13) investigated many seeds and finally concluded that saccharose or a nearly related sugar was derived from oils without the glycerine being set free, as in ordinary saponification. MILLER (15), in his studies on the sunflower, records the gradual disappearance of reserve oil and protein material from the cotyledons, with the increase of sugar and protein-free nitrogen in the hypocotyl and roots. In 1912 IVANOW (10) followed the transformation of the oils in seeds during germination. He chose for his work seeds having saturated fatty acids, and others having unsaturated fatty acids. The unsaturated fatty acids were found to be transformed first, and later the saturated fatty acids were used. He ascribed the fall in the iodine number of the fats to the more rapid transformation of the unsaturated fatty acids to carbohydrates, and not to the formation of acids of shorter chains.

KOSSEL (12), as early as 1891, believed lecithin to be present in all protoplasts. STOKLASA (21) states that the phosphatides of rape seeds during five days' germination increased from 0.45 to 5.22 per cent. The dry beet seed with a phosphatide content of 0.45 was found to contain 1.78 per cent after nine days' germination. CZAPEK (2) quotes data from SCHULZE and his school, also others, showing the general distribution and percentage of phosphatides in plant tissues. FRANKFURT (6) in 1894 studied the seeds and

seedlings of the sunflower, and found that glutamin and asparagin increased during germination. PALLADIN (18) in 1896 stated that an increase of the proteins indigestible in gastric juice (nearly proportional to the amount of nucleo-proteins) accompanied the germination of wheat in darkness. ZALIESKI (22) in 1911 reported the general appearance of nucleo-protein in plant tissues. He also found an increase of nucleo-protein with the germination of wheat and corn seeds.

Investigation

CULTURE METHODS

After the seeds had been prepared, as described in an earlier paper (17), they were placed on moist filter paper in Petri dishes and subjected to a temperature of about 5°C. in darkness for after-ripening and germination. Distilled water was added at intervals to keep them moist. The changes occurring had to do with the reserve material already in the seed. The after-ripened seeds and seedlings were kept under these conditions until being prepared for analysis. Analyses were made at three different stages: dry seeds, after-ripened seeds, and late seedling development.

DRY SEEDS.—The hard coats were removed from the dried seeds before preparing them for analysis. In this, as well as in the following stages, the embryo and endosperm (or more exactly, the nucellus, megaspore membrane, and all parts surrounded by these two structures) were the parts analyzed.

AFTER-RIPENED SEEDS.—The seeds were found to require 100 days' storage in a germinator at about 5°C. for after-ripening (or to become ready for immediate germination). The seed at this particular period has already split open the hard coat, and the hypocotyl is breaking through the nucellus. It was at this time that the after-ripened seeds were removed from the hard coats and prepared for the analysis.

DEVELOPED SEEDLINGS.—It required 35 days at 5°C. for the after-ripened seeds to become developed seedlings, which were between 3 and 4 cm. long. The cotyledons were extended and free from the old endosperm, nucellus, megaspore membrane, etc. These latter structures were collected and put with the seedlings

so as to have comparable analytical results. The hard coats were separated and discarded, just as in the collection of the seed material.

Analytical methods and results

The material for analysis was prepared according to Lowenstein (14) and Miller (15), except for slight modifications. The collected seeds and seedlings were thoroughly ground with 95 per cent alcohol in a mortar; then the material was transferred to evaporating dishes and the alcohol evaporated. After thus treating the material three times with 95 per cent and twice with absolute alcohol, it was dried in a vacuum at 75°C. for one hour. The material was then powdered and placed in the desiccator until analyzed. When analyzed the material was in perfect condition, and showed no signs of oxidation.

The method followed in the analysis was outlined by Koch (11). As it was necessary to make both fat and protein analysis on the same sample, the acid precipitation of the lipoid fraction, as earlier described by Koch, could not be used because of possible protein hydrolysis. The lipoids, therefore, were extracted by an 18-hour continuous extraction with hot absolute anhydrous ether. Calcium chloride tubes were used to protect the material from moisture during the extraction. Lipoid or ether soluble material is referred to as F_1. The whole of the lipoids were not dried to get the true weight, because of the danger of oxidizing the unsaturated compounds. This lipoid weight was derived by subtracting the weight of the dry lipoid-free material from the original dry weight. Such a change made it possible to analyze the lipoids at once and avoid oxidation (table II); then the lipoid-free material was extracted with hot 50 per cent alcohol for 12 hours. This 50 per cent alcohol soluble material is indicated as F_2, or extractives. These extractives were dried to constant weight in vacuum, dissolved in hot water, and portions taken for the analysis. The 50 per cent alcohol insoluble material (or F_3) was dried in vacuum, weighed, powdered, and portions taken for analysis (table IV).

Table I gives some general data. The amount of water and solid material found in the air-dry seeds, after-ripened seeds, and seedlings at the time the material was prepared for analysis, is

given as percentage of seed weight with hard coats removed. Total nitrogen is given as percentage of total dry substance and was obtained by the KJELDAHL method. The analysis of chlorophyll

TABLE I

MATERIALS	DRY SEEDS			AFTER-RIPENED SEEDS		SEEDLINGS	
	a	b	c	a	b	a	b
Water	7.19	7.25	7.15	52.64	53.01	88.38	88.54
Solid material	92.81	92.75	92.85	47.36	46.99	11.62	11.46
Chlorophyll as depth of color	.....	.....	.....	Traces	Traces	100.00	100.00
Dry weight of total nitrogen	5.70	.....	.....	5.70	.....	5.72	.....

could not be attempted. The amount of chlorophyll present, however, is given as percentage, and was estimated from the depth of color, considering the chlorophyll content of the seedlings as 100 per cent.

TABLE II

LIPOIDS (F_1)

MATERIALS	DRY SEEDS		AFTER-RIPENED SEEDS		SEEDLINGS	
	a	b	a	b	a	b
Total lipoids as percentage total dry weight	53.60	53.69	43.93	44.01	11.72	11.00
Phosphatides as percentage total dry weight	1.23	1.22	2.80	2.84	2.36	2.37
Acid value as percentage ether extract	1.97	1.90	5.68	5.09	27.75	28.12
Saponification number	174.7	172.3	178.3	180.0	126.0	127.1
Iodine number	133.6	135.0	132.1	131.0	121.4	122.0
Neutral fats as percentage ether extract	95.73	95.82	87.93	88.46	52.07	50.01
Percentage of P in total dry weight	0.03	0.03	0.109	0.110	0.092	0.095
Percentage N as percentage of total dry weight	0.01	.....	0.05	.....	0.05	.....
Percentage of increased weight due to probable O_2 absorption	9.9	.....	11.1	.....	2.1	.....

In tables II, III, and IV the amount of substance found has been given as percentage of the total dry substance unless otherwise stated. Thus in table II the acid value, saponification number, iodine number, and neutral fats were determined for the total lipoid

fraction. The percentage of phosphatide was estimated from the lipoid; P times the factor 25.77. WIJ's iodine solution was used in the determination of the iodine number. No direct nitrogen determination was made on the lipoid fraction. The percentage of nitrogen given was found by subtracting the extractive and protein nitrogen (tables III and IV) from the total nitrogen given in table I. Table II also gives the percentage of oxygen taken up by the lipoid material under artificial oxidation.

TABLE III

EXTRACTIVES (F_2)

PERCENTAGES OF TOTAL DRY WEIGHT	DRY SEEDS			AFTER-RIPENED SEEDS			SEEDLINGS		
	a	b	c	a	b	c	a	b	c
Total extractives....	6.68	6.50	....	15.24	15.55	....	34.02	34.59	....
Total nitrogen.....	0.13	0.12	....	1.04	1.10	....	2.05	2.11	....
Ammonia nitrogen..	0.0004	0.0004	....	0.0004	0.0003	....	0.0001	.....	....
Amino acid nitrogen.	0.04	0.05	0.04	0.27	0.25	0.30	0.92	0.95	0.93
Reducing sugars after hydrolysis.....	1.27	1.30	1.25	1.99	1.80	1.88	7.49	7.43	7.55
Direct reducing sugars.....	Traces	.....	....	0.07	.....	0.05	1.35	1.34	1.37
Reducing sugars after removal of tannins.....	Traces	.....	....	0.69	.....	....	.....	.....	....
Pentose reaction....	0	.....	....	Marked	.....	....	Very marked	.....	....
Unaccounted for material (organic acids).....	.....	.....	....	2.7	.....	....	9.7	.....	....

Table III gives the extractives as percentage of total dry substance. The total nitrogen was determined by the BOCK and BENEDICT (1) modification of the FOLIN-FARMER (5) procedure. Ammonia nitrogen was determined by the same procedure after aerating under diminished pressure a large part of the extraction solution made slightly alkaline and collecting the ammonia in dilute HCl. The undistilled material was neutralized with acetic acid, concentrated, and used for van Slyke amino nitrogen determinations. A third portion was used for the sugar determinations. The tannins were removed with pure casein. Both after-ripened seeds and seedlings gave indications of pentose.

The proteins, polysaccharides, etc., are given in table IV as percentages of total dry substance. The protein nitrogen, which is stated as percentage of protein, was determined by the KJELDAHL procedure. Polysaccharides were determined by the MONSON-WALKER and BERTRAND method. This material gave very marked pentose reactions. The cellulose was not determined as such.

TABLE IV

PROTEINS, POLYSACCHARIDES, ETC. (F_3)

PERCENTAGES OF TOTAL DRY WEIGHT	DRY SEEDS		AFTER-RIPENED SEEDS			SEEDLINGS	
	a	b	a	b	c	a	b
Proteins, polysaccharides, etc..	39.73	39.00	40.83	40.43	.....	54.25	54.49
Total proteins ($F_3 \times 6.25$).....	34.21	34.10	28.50	27.95	.....	22.31	22.70
Total hydrolyzable sugars.....	0.0	0.0	0.2	0.23	0.19	14.91	14.79
Indications of pentoses.....	0.0	0.0	Marked	.....	.....	Very marked	.....

Discussion

These results force upon one's attention the great constructive changes as compared with the destructive changes. The major fractions seem to be well accounted for. Such a condition can only be understood when one considers that these results deal with a seed that requires long continued after-ripening and germination at a very low temperature. Although the seed material was kept at a temperature of about 5°C., the constructive metabolism went on at a rapid rate. The digestion of storage fats and proteins was accompanied by the synthesis of many formative and metabolic compounds. The rate and extent to which these changes were carried on even at 5°C. prove the power and efficiency of enzyme action. This low temperature, by retarding respiration, reduced the combustion of materials to a minimum, and thereby favored the accumulation of formative materials in the cells. This accumulation of cell building and cell active materials, together with the culmination of enzymes, probably leads to the after-ripening of dormant organs.

The lipoids decreased 9.7 per cent during after-ripening, and 32 per cent during the seedling development. It will be seen that

the neutral fats sustained this loss. The respiration occurring during the after-ripening period amounted to only 2 per cent, while during the seedling period it amounted to about 5 per cent of the total dry weight. This small amount of material used by the respiration compared with the large amounts of formative, storage, and structural material, high in oxygen content, made from the apparently small amounts of fats, low in oxygen content, will easily account for the low respiratory quotient reported in an earlier paper (17) for these seeds during germination.

The phosphatides more than doubled during the after-ripening process. Glycerine and fatty acids were supplied by the hydrolysis of fats, while phosphoric acid and nitrogen-containing complex were probably derived from inorganic phosphorus and the protein hydrolysis which accompanied the after-ripening. A slight decrease in the amount of phosphatides occurred during seedling development. This decrease could represent the phosphoric acid necessary for the formation of the nucleic acid, which was constructed at this period.

The acid value of the ether extract increased during both after-ripening and seedling development. The iodine number decreased, while the saponification increased slightly without a marked appearance of carbohydrates. Such a condition would probably accompany the breaking up of long carbon chains into shorter chained compounds. The increased fall in the iodine number during the seedling development was due perhaps to the more rapid transformation of unsaturated fatty acids to carbohydrates (10). This carbohydrate accumulation during seedling development amounted to 20 per cent (tables III and IV). The saponification number reached a minimum value for the seedlings, indicating a large percentage of long chained fatty acids. This is accounted for by the large percentage of phosphatides in the seedling lipoids. It appears that these values change materially in the same tissues with different stages of development.

Dry seed and after-ripened seed lipoids were made to take up respectively 9.9 and 11.1 per cent of increased weight due to probable oxygen absorbed by artificial oxidation. There was a slight increase in the reducing power of the lipoids during after-ripening.

Under the same conditions the seedling lipoid material increased in weight only 3.1 per cent due to oxygen absorption.

Of considerable interest is the increase in extractives with after-ripening and seedling development. This is represented by increasing amounts of amino acid nitrogen, and other forms which probably represent amides, peptides, nucleic acid derivatives, alcohols, etc. It also represents increased amounts of various sugars, and very probably organic acids. The ammonia nitrogen value did not change during after-ripening, although it did decrease during the seedling development. This decreasing amount corresponds to the amount of nitrogen required during this same period to build the chlorophyll. As ammonia plays such an important part in the synthesis of proteins (amino acids), however, it is probable that this decrease is of no significance and that the amounts fluctuate. In connection with this it is evident that some proteins, having carbohydrate groups, were rebuilt during the after-ripening and especially the seedling development. Amino nitrogen, van Slyke method, increased about sevenfold during the after-ripening period, and over threefold again during the seedling period (table III). The Formol titration on similar lots of seeds showed a like increase of amino acids during the after-ripening period. The ratio of the amino nitrogen to the total nitrogen of F_2 is as follows: dry seeds one-third, after-ripened seeds one-third, and seedlings one-half. This could mean the formation of shorter-chained amino compounds or the further digestion of peptides, proteoses, or peptones. The increasing amount of non-amino nitrogen during after-ripening and seedling development shows the accumulation of other nitrogenous compounds. This is very probably represented by nucleic acid, peptides, peptones, amides, and other extractives.

Although the sugar formation was very meager during the after-ripening period, it reaches noticeable proportions during the germination and seedling development. Table III shows a 0.5 per cent increase of reducing sugars, after hydrolysis, for the after-ripened seeds. This included a few hundredths per cent of direct reducing sugar. It is evident that nearly all of the reducing sugar of the dry and after-ripened seeds is tied up with the tannins.

The dry seeds gave no pentose reactions, while the after-ripened seeds and seedlings gave marked reactions. During the seedling development the percentage of sugars increased manyfold.

Comparing the amount of total extractives and the sum of the analyzed fractions, it will be seen that there is considerable material unaccounted for. After adding an average percentage for ash, however, the 6.68 per cent of extractives for the dry seeds is nearly all accounted for. After adding the same amount for ash in the after-ripened seeds and the seedlings, there remain respectively 3 and 10 per cent of the extractives unaccounted for. It is evident that this material is not proteins or, much less, decomposition products of the same. Such an explanation would require a protein factor of ten or more. It could not be due to an increase of ash because the seeds were kept in distilled water cultures. The sugars by no means account for this unknown material, and a possible explanation is the presence of organic acids. A review of the analytical results of tables II, III, and IV also shows that there is no other way to account completely for the disappearance of so much fat. It is evident, therefore, that at least part of the fatty acids were oxidized to other organic acids. In the course of the analysis (when F_2 was neutralized for ammonia distillation) it was found that the extractives for dry after-ripened seeds and seedlings all gave an acid reaction. No acid had been used thus far in the analysis, and this acidity was evidently due to acids in the tissues. The extractives of the dry seeds were distinctly acid, while the extractives of the after-ripened seeds and seedlings were very acid. It was also noted that more N/10 NaOH was used to neutralize the seedlings than either the dry seeds or after-ripened seeds. These, with previous results, point to the accumulation of organic acids.

Table IV shows an increase of the protein polysaccharides fraction during after-ripening and germination. There was a decrease in the proteins with an increase of starch. During after-ripening, however, there was a 6 per cent decrease of proteins with only a 0.2 per cent increase of starch. Of course much of this protein material appears in F_2 as amino acids and other nitrogenous compounds. Moreover, some proteins after hydrolysis and deami-

nation very probably gave rise to sugars and acids or were respired. The pentose reactions indicate the rebuilding of proteins with carbohydrate groups. During the germination and seedling development the proteins were hydrolyzed to give rise to the amino acids and nitrogenous compounds of F_2, with the formation of some carbohydrates. From the amount of starch (table IV) and sugars (table III) appearing in the seedlings and the carbohydrates required for cellulose structure, it is evident that not only the proteins but still more the fats contribute to the formation of these materials. From the constant quantity of nitrogen in the analysis and the fact that no nitrogen compounds were added, it is evident that the chlorophyll nitrogen was derived from other nitrogenous compounds.

Summary

A review of these results, together with the changes reported in the previous paper (17), give an idea of the many changes accompanying the after-ripening of dormant organs. These changes are represented by the accumulation of cell building materials: acids, phosphatides, active reducing substances, soluble sugars, pentoses, amino acids, soluble proteins, and other nitrogenous compounds; the accumulation of enzymes; the dispersion of materials; and the transformation of storage materials. This rapid accumulation of simple plastic cell materials coupled with minimum respiration and combustion of materials probably forces the dormant organs to activity. One thus sees the awakened active organ as a very unstable structure made up of many unstable compounds. If these changes are not the basis of the after-ripening process, they are found to accompany the after-ripening process.

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